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Small intestine

Structural and functional changes of the duodenum in human norovirus infection

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ABSTRACT

Background: Norovirus infection is the most frequent cause of infectious diarrhoea in the western world. This study aimed to characterise functionally and histomorphologically the diseased duodenum in human biopsies.

Methods: Norovirus infection was diagnosed by the Kaplan criteria and confirmed by PCR of stool samples. Duodenal biopsies were obtained endoscopically. In miniaturised Ussing chambers, short circuit current, flux measurements and impedance spectroscopy were performed. Histological analysis including apoptosis staining and characterisation of intraepithelial lymphocytes was performed. Tight junction proteins were quantified by immunoblotting.

Results: In norovirus infection, epithelial resistance decreased from (mean (SEM)) 24 (2) Ω cm^2 in controls to 10 (1) Ω cm^2. Mannitol flux increased from 113 (24) nmol h^{-1} cm^{-2} in controls to 242 (29) nmol h^{-1} cm^{-2}. Microdissection revealed a villus surface area reduced by 47% (6.6%). Intraepithelial

lymphocytes were increased to 63 (7) per 100 enterocytes, with an increased rate of perforin-positive cytotoxic T cells. Expression of tight junctional proteins occludin, claudin-4 and claudin-5 was reduced. The epithelial apoptotic ratio was doubled in norovirus infection. Furthermore, the basal short circuit current was increased in norovirus infection and could be reduced by bumetanide and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB).

Conclusions: Norovirus infection leads to epithelial barrier dysfunction paralleled by a reduction of sealing tight junctional proteins and an increase in epithelial apoptosis, which may partly be mediated by increased cytotoxic intraepithelial lymphocytes. Furthermore, active anion secretion is markedly stimulated. Thus, the diarrhoea in norovirus infection is driven by both a leak flux and a secretory component.

Human norovirus is the most frequent cause of epidemic non-bacterial gastroenteritis worldwide.[1] The norovirus was first described by Albert Kapikian in 1972 who visualised the virus by immune electron microscopy of passed stool filtrate from an outbreak in Norwalk, Ohio.[2] The disease is characterised by a rapid onset of nausea, vomiting and watery diarrhoea which is usually self-limiting in the immunocompetent host.[3] However, there are reports of outbreaks in hospitals that led to long-lasting and life-threatening illness in immune-compromised patients.[4] Besides faecal–oral spread and contaminated water or food as a source of infection, viral transmission also occurs by aerosols after vomiting. Diagnosis of norovirus infection is primarily based on clinical features; however, molecular analysis using PCR techniques allows rapid testing for infection in stool samples or vomitus, and is considered as gold standard for diagnosis.[5]

Despite much effort over the last 40 years, for a long time it has not been possible to replicate the virus and only very recently are there first reports of viral replication in tissue culture.[6 7] Therefore, insight into the pathophysiology of this disease is limited. Early studies in the 1970s of experimental norovirus infection of healthy volunteers resulted in the first histopathological description of the small intestine, showing a rapid and completely reversible blunting of the villi, an infiltration with mononuclear cells and a shortening of microvilli with reduced activity of brush border enzymes.[8 9] Besides these findings from experimental human infection, the ability to express virus-like particles that morphologically and immunologically resemble the virus led to insight into norovirus attachment to human epithelial cells and to aspects of the immune response to this pathogen. Over the last decade it has become evident that some virus strains require histo-blood group antigens as a host receptor which are present on the epithelium.[10] Furthermore, an animal infection model using the murine norovirus (MNV-1) revealed a tropism of MNV-1 to macrophages and dendritic cells, and the small intestine to be the primary site of viral replication. However, it should be noted that wild-type mice display no clinical signs of infection at all and in the immune-compromised background it is a systemic and fatal disease with virtually no signs of gastroenteritis. Thus, the transferability of these findings to human disease is rather limited.[11–13] More recently, norovirus pathogenesis in a gnotobiotic pig model was described, showing a rapid and self-limiting diarrhoeal disease.[14] Here, norovirus was detected in enterocytes of villi in the proximal small intestine. The histopathological changes though were rather subtle, with enterocyte apoptosis as the prominent feature.

Therefore, we started to study norovirus infection in human small intestinal biopsies and we present here combined morphological, functional and molecular biological data of this frequent gastrointestinal infectious disease.

MATERIALS AND METHODS

Study subjects

Seven immune-competent patients with clinical signs of acute norovirus infection (acute onset of vomiting and watery diarrhoea (>5/day)) who were referred to the Charité Campus Benjamin Franklin University Hospital in Berlin and underwent upper gastrointestinal endoscopy were included. Endoscopy with duodenal biopsies was performed as routine endoscopy to exclude other causes of diarrhoea (such as, for example, giardiasis) and other causes of abdominal pain and vomiting (such as, for example, peptic ulcer disease), respectively. Endoscopy was performed 0–6 days (median 3 days) after onset of symptoms. Diagnosis of norovirus infection was confirmed by PCR of stool samples as described below. A group of seven patients who underwent endoscopy for other reasons (abdominal pain, suspected malignancy, rectal bleeding or iron deficiency) without macroscopic or microscopic signs of pathology served as controls. All patients gave their written consent to undergo biopsies for scientific purposes, and the study was approved by the local ethics committee. A miniaturised Ussing chamber with an exposed area of 0.049 cm^2 was used as described previously.[15] Briefly, measurements were performed on duodenal specimens which were obtained from the distal duodenum during upper endoscopy by a forceps biopsy. Biopsies were fixed on a disc with a opening diameter of 3.4 mm using histoacryl tissue glue. The time between taking the biopsy and mounting into the Ussing chamber was ~20–30 min, during which the specimen was kept in oxygenated phosphate-buffered saline on ice. After an equilibration period of 20 min, impedance spectroscopy and flux measurements were done, and subsequently pharmacological experiments with bumetanide and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were performed.

Stool analysis

RNA was extracted from 140 µl of the 10% faecal suspension by a spin column technique using the QIAampViral RNA Mini Kit (Qiagen, Hilden, Germany). One-tenth of the extracted RNA was subjected to open reading frame 1 reverse transcription (ORF1 RT)/nested PCR[16 17] and to multiplex real-time PCR.[18]

Solutions and drugs

The bathing solution of flux experiments contained the following (in mmol/l): Na^+ 140, Cl^- 123.8, K^+ 5.4, Ca^{2+} 1.2, Mg^{2+} 1.2, HPO_4^{2-} 2.4, $H_2PO_4^-$ 0.6, HCO_3^- 21, D(+)-glucose 10, β-OH-butyrate 0.5, glutamine 2.5, D(+)-mannose 10 and mannitol 10. The solution was gassed with 95% O_2 and 5% CO_2. The temperature was maintained at 37°C using water-jacketed reservoirs, and the pH was 7.4 in all experiments. Antibiotics (50 mg/l piperacillin and 4 mg/l imipenem) served to prevent bacterial growth and had no effect on short circuit current (I_{sc}) in the concentrations used. NPPB and bumetanide were purchased from Sigma Chemicals (St Louis, Missouri, USA).

I_{SC} and tracer flux measurements

Ussing-type experiments were performed as described previously using a computer-controlled voltage clamp device (CVC 6; Fiebig, Berlin, Germany).[15 19] Paracellular permeability was determined by [^{3}H]mannitol flux (mucosa to serosal) under short circuit conditions. Briefly, 10^7 cpm of labelled mannitol (specific activity 20 Ci/mmol; ARC, St Louis, Missouri, USA) was added to the mucosal side. After an equilibration period, a fraction of the serosal volume was sampled over three 15 min intervals and analysed using a β-counter. Inhibition of anion secretion was achieved by bumetanide (10^{-5} mol/l) added to the serosal side or by NPPB ($5x10^{-4}$ mol/l) added to both sides.

Histology

Biopsy specimens from the duodenum were immediately fixed in 10% buffered formalin, and embedded in paraffin for morphological analysis. Serial sections (3 µm) were stained with H&E or dewaxed for fluorescence detection of epithelial apoptoses. Cellular DNA was stained with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay (Roche, Mannheim, Germany). For immunostaining, 4 µm thick sections were cut, deparaffinised and subjected to a heat-induced epitope retrieval step before incubation with antibodies. Sections were immersed in sodium citrate buffer solutions at pH 6.0 and heated in a high-pressure cooker. The slides were rinsed in cool running water, washed in Tris-buffered saline (pH 7.4) and incubated with primary antibodies. The primary antibodies included the monoclonal antibodies against CD3 (clone UCHT1, dilution 1:25; Dako, Glostrup, Denmark), CD4 (1F6, 1:25; Novocastra Laboratories, Newcastle upon Tyne, UK), CD8 (C8/144B, 1:100; Dako), Ki-67 (MIB-1, 1:2000; Dako), perforin (P1-8, 1:500; Holzel, Cologne, Germany) and cleaved caspase-3 (Asp175, 1:200; Cell Signaling, Danvers, Massachusetts, USA). For detection, the alkaline phosphatase–antialkaline phosphatase complex (APAAP) or avidin–biotin conjugate (ABC) method using the streptavidin AP kit or biotinylated rabbit antirat antibody (Dako) was applied. Negative controls were performed by omitting the primary antibodies. The number of positive intraepithelial lymphocytes (IELs) or the rate of epithelial apoptosis was determined per 100 enterocytes.

Western blot analysis

To determine tight junction protein expression, western blot analysis was performed from membrane extracts of duodenal biopsy specimens. Tissues were homogenised (by douncing) in iced lysate buffer containing 20 mmol/l Tris, pH 7.4, 5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 0.3 mmol/l EGTA, 1 µl/ml aprotinin, 16 µg/ml benzamidine HCl, 10 µg/ml phenanthroline, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mmol/l phenylmethylsulfonyl fluoride, 210 µg/ml sodium fluoride, 2.16 mg/ml β-glycerophosphate, 18.5 µg/ml $NaVO_4$, and 1 µl/ml trypsin inhibitor (all substances obtained from Sigma, St Louis, Missouri, USA). Membrane fractions were obtained by passage through a 26 Gx½ inch needle. To remove insoluble material, the extract was centrifuged at 200 *g* for 5 min at 4°C. The supernatant was then centrifuged at 43 000 *g* for 30 min at 4°C. The pellet representing a crude membrane fraction was resuspended in lysate buffer. Protein concentrations were determined by Pierce BCA assay (Pierce, Rockford, Illinois, USA). Aliquots of 5 µg of protein were separated by polyacrylamide gel electrophoresis (8.5% for occludin and 12.5% for claudins) and transferred to a polyscreen polyvinylidene difluoride transfer

membrane (NEN Life Science Products, Boston, Massachusetts, USA). Blots were blocked for 2 h in 5% milk powder and then overnight in 5% bovine serum albumin (at 4°C) before incubation with primary rabbit polyclonal immunoglobulin G antibodies against claudin-1, -2, -3 and -5, and occludin, and primary monoclonal antibody against claudin-4. Peroxidase-conjugated goat antirabbit/antimouse immunoglobulin G antibodies and the chemiluminescence detection system Lumi-LightPLUS Western blotting kit (Roche, Mannheim, Germany) were used to detect bound antibodies. Antibodies were provided by Zymed Laboratories (South San Francisco, California, USA). Chemiluminescence signals were detected using an LAS-1000 imaging system (Fuji, Tokyo, Japan) and analysed with the AIDA program package (Raytest, Berlin, Germany).

Statistical analysis

Results are given as means (SEM). Significance was tested by the two-tailed Student t test. A p value <0.05 was considered significant.

RESULTS

Impedance spectroscopy and mannitol flux

Representative original impedance locus plots of duodenal specimens from a patient with norovirus infection and a control patient are shown in fig 1. Statistical evaluation of this analysis is presented in table 1 and revealed a >50% decrease of epithelial resistance in specimens from patients with norovirus infection when compared with control values. The subepithelial resistance was also affected, which contributed to the substantial decrease in transmural wall resistance (table 1). Likewise, mannitol flux in norovirus-infected specimens increased by >100% compared with control duodenum (table 1). There was a strong correlation between epithelial resistance and mannitol flux ($r = –0.8$; $p<0.01$), but only a weak correlation between epithelial resistance and I_{sc} ($r = –0.6$; $p<0.05$).

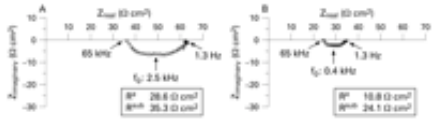

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Figure 1 Epithelial barrier dysfunction in norovirus infection. Original impedance locus plots of human duodenum of control (A) and norovirus infection (B). Z_{real} gives the ohmic component and $Z_{imaginary}$ the reactive component of the complex impedance. Intersections between the semicircle and *x*-axis at low and high frequencies represent transmural wall resistance (R^t) and subepithelial resistance (R^{sub}), respectively. R^t minus R^{sub} represents the epithelial resistance (R^e). For a detailed explanation of one-path impedance spectroscopy, see Gitter *et al*.[20]

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Table 1 Epithelial resistance and mannitol permeability in human norovirus infection

Conventional histology, immunohistochemistry and epithelial apoptosis

Mucosal architecture as judged by conventional histological H&E-stained thin sections did not show any gross lesions such as erosions or ulcers (fig 2B). In 3 out of 7 cases, minimal villus blunting was observed. However, more refined analysis by using a microdissection technique revealed a reduction of villus surface area as the result of a reduction in villus height, whereas crypt length was not significantly altered in norovirus infection (table 2).

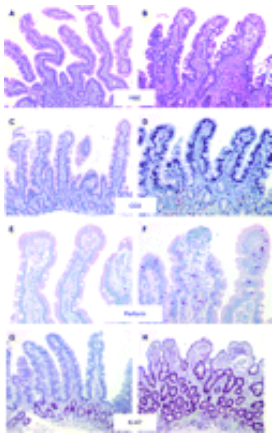

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Figure 2 Histological analysis of the duodenum in norovirus infection. Representative sections of biopsy specimens from the duodenum of patients with norovirus infection (right side) are shown stained with H&E or immunostained with anti-CD3, antiperforin and anti-Ki-67, respectively. Specimens show slight blunting of villi (see also table 2), an increased number of intraepithelial $CD3^+$ T cells with a high proportion of T cells with a cytotoxic phenotype and an increase in Ki-67-positive epithelial cells as compared with normal duodenal mucosa with long and slender villi, low number of intraepithelial $CD3^+$ T cells without expression of the cytotoxic molecule perforin and a very low Ki-67 proliferative index at the base of the crypts (for quantification see table 3; magnification x20).

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Table 2 Mucosal morphometry in norovirus infection

IEL count as obtained in anti-CD3-stained sections was markedly increased compared with controls (table 3 and fig 2D). To characterise IELs in more detail, anti-CD8, CD4 and perforin staining was performed. As expected, the vast majority of $CD3^+$ IELs were $CD8^+$ T cells (table 3), whereas $CD4^+$ T cells in the IEL compartment were only rarely observed (data not shown). Further characterisation of these $CD8^+$ T cells revealed an increased proportion of cytotoxic T cells characterised by the

expression of the cytotoxic molecule perforin (table 3; fig 2F). Staining against Ki-67 antigen showed an enlarged proliferation zone in norovirus-infected duodenum with a significant increase in Ki-67-positive epithelial cells (table 3; fig 2H).

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Table 3 Immunohistological analysis of duodenum in human norovirus infection

The apoptotic ratio was determined using TUNEL and anticaspase-3-stained sections. Typical apoptotic changes associated with epithelial apoptosis comprise condensation of chromatin, its compaction along the periphery of the nucleus and segmentation of the nucleus in conjunction with a positive TUNEL or anticaspase-3 staining, respectively. With both methods, the epithelial apoptotic ratio was more than doubled in norovirus-infected tissues when compared with control tissues (fig 3).

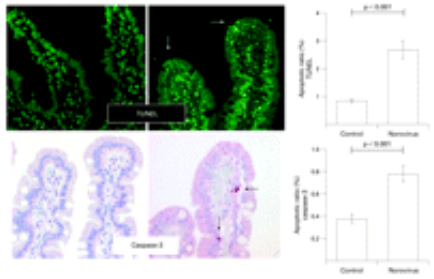

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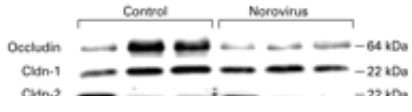
Figure 3 Epithelial apoptosis. Detection of epithelial apoptoses by staining of a thin section of duodenal specimen with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) and anticaspase-3 (original magnification x20). Apoptoses are depicted by arrows, with no positive cells in normal controls in these selected sections. On the right, quantitative analysis of the respective staining technique shows an increase in epithelial apoptoses in norovirus-infected duodenum (n = 7; $p<0.001$).

Tight junction protein expression

To investigate further whether altered tight junction structure contributes to the decreased R^e in norovirus infection, immunoblot analysis was performed for the tight junction proteins occludin and claudin-1, -2, -3, -4 and -5. Because these proteins are integral membrane proteins forming tight junction strands, crude membrane fractions were used. The data on tight junction protein expression are given in fig 4, showing reduced expression of occludin, claudin-4 and claudin-5 compared with control levels.



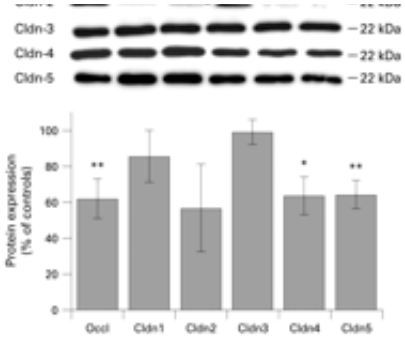


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Figure 4 Tight junctional protein expression. Expression of occludin (Occl) and claudin (Cln)-1, -2, -3, -4 and -5 was determined in crude membrane fractions by immunoblotting. Statistical analysis is based on densitometries from six different patients with norovirus infection (expressed as a percentage of the controls on the same immunoblot). Data are given as the mean (SEM). *p<0.05, **p<0.01.

Short circuit current measurements

Another important aspect of the impedance spectroscopy is the correction of active transport rates for subepithelial resistance (R^{sub}) contributions. Whenever significant non-epithelial series resistances are present between the voltage-sensing electrodes in addition to the R^e, measured I_{sc} or net fluxes have to be corrected for the contribution of these resistances. This correction is well known when applied to bath resistance, but it is also necessary to correct I_{sc} and net fluxes for the R^{sub} of intestinal preparations. The implications of this correction have been given elsewhere in detail.[19–23] Generally, after correction for the bathing solution, the true active transport rate becomes underestimated by a factor that is given by the ratio of R^t over R^e. This factor was 3.3 (0.2) in patients with norovirus infection and 2.4 (0.1) in controls ($p<0.01$; table 1). Because these factors significantly differed by ~40%, I_{sc} values of both groups were compared only after performing this correction (table 4).

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Table 4 Active epithelial transport in norovirus infection

Basal I_{sc} was more than doubled in norovirus infection (fig 5, left). Inhibitory experiments with bumetanide and NPPB to characterise the increase in I_{sc} further revealed an increased proportion of bumetanide- and NPPB-sensitive I_{sc}s in norovirus infection, pointing to an increase in active electrogenic chloride secretion in this disease (fig 5, right). Furthermore, there was an increased phlorizin-sensitive I_{sc} in norovirus infection (table 4). This was suprising since absorption takes place in the morphologically compromised villus. In any case, this finding indicates that there is no malabsorptive component in this type of diarrhoea. The increase in phlorizin-sensitive current may even indicate some type of adaptative mechanism of the mucosa in this state of diarrhoea. However,

clinically this adaptation may not be relevant due to the pronounced disturbance of oral nutrient intake in the acute phase of the disease.

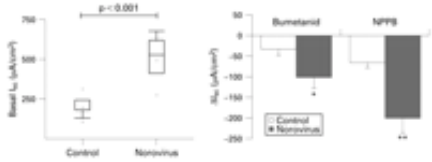

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Figure 5 Epithelial transport in norovirus infection. Short circuit current (I_{SC}) of human duodenal specimens obtained with the Ussing technique under basal conditions (left) and change of current after addition of bumetanide and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (right). Values were corrected for subepithelial resistance contributions as described in the Materials and methods section. Data are the mean (SEM). n = 6 (norovirus). n = 7 (control). *p<0.05, **p<0.01.

DISCUSSION

Although norovirus infection has caused numerous outbreaks in the recent past, very little is known regarding morphological and functional changes of the human intestinal mucosa. Therefore, we investigated tissue samples obtained from patients with acute norovirus infection and performed detailed immunohistochemical and electrophysiological studies of the epithelial barrier.

As the first important finding from this duodenal biopsy study of human norovirus infection, a reduction of villus surface area was detected which was paralleled by the appearance of a dense intraepithelial immune cell infiltrate of $CD8^+$ lymphocytes. This is largely in agreement with previous histological data obtained from experimental human infection which describe an increase of mononuclear cells in the lamina propria of small intestinal biopsies. However, in one of the publications it is also noted that there is "increased infiltration of mononuclear cells in the intercellular spaces between epithelial cells", a cell population which is now called IELs.[8 9] This cell type belongs to a unique T cell population which is interspersed between epithelial cells of both the small and large intestine, the function of which especially in norovirus infection is largely unknown.[24] Data from isolated human IELs suggest that this cell type in general possesses lytic ($CD8^+$) or cytokine-producing ($CD4^+$) properties. However, most of the IEL population express CD8 as we could also show here for norovirus infection.[25 26] One differential diagnosis of increased IELs are viral infections of the gastrointestinal tract. However, to our knowledge, this has not been analysed in human norovirus infection so far and it was surprising to find such a high number of IELs in this disease. Therefore, norovirus infection has to be included among the conditions that are associated with increased numbers of IELs (eg, coeliac disease, autoimmune enteropathy, *Helicobacter pylori* infection and giardiasis).

Furthermore, the increased perforin staining of the IELs in norovirus infection may even suggest a contribution to the epithelial damage via epithelial apoptosis that leads to villus blunting and epithelial

barrier dysfunction, although part of these alterations are assumed to result from direct viral infection of the enterocytes (see below). The significance of this process is also underlined by the fact that villus surface area reduction takes place despite a significant stimulation of epithelial proliferation (= hyper-regenerative villus "atrophy") which has already been described previously in this disease after experimental infection.[23] In these longitudinal studies on healthy volunteers, the dynamics of the epithelial changes have been elucidated by histological analysis before, at the maximum of infection and 2 weeks after infection in the reconvalescence phase where the histological appearance had already completely returned to normal. Obviously, norovirus infection leads to a rapid and short-lived morphological mucosal alteration. Rapid villus height reduction has also been shown in another viral infection, the simian immunodeficiency virus (SIV) infection model.[27] Here, these changes are thought to be mediated by cytotoxic lymphocytes and also by activation of matrix metalloproteinases. In this model, the changes were visible at 1 week postinfection and there were no longer any morphological alterations at 6 weeks postinfection. In conclusion, these findings suggest that the mechanism of rapid villus height reduction/atrophy may largely be dependent on activation of IELs with a direct cytotoxic or cytokine-mediated effect on enterocytes.

As a further important result of the electrophysiological analysis severe epithelial barrier dysfunction became evident. This can contribute to the diarrhoea during norovirus infection by a leak flux mechanism—that is, ions and water "leak back" from the subepithelial capillaries into the intestinal lumen by paracellular diffusion due to the increased permeability of the tight junction. This is not a unique feature in the norovirus-infected intestine but has also been observed before for other enteropathogens such as, for example, for *Giardia lamblia* and in HIV enteropathy, using the same technique.[16 28] Furthermore, it is an important feature in *Clostridium difficile*-induced colitis.[29] Direct evidence for the concept that a barrier disturbance contributes to (infectious) diarrhoea via a leak flux mechanism results from the work of Fasano *et al*.[30] In that work, attenuated *Vibrio cholerae* strains depleted of the chloride secretion-inducing cholera toxin gene still induced diarrhoea in human volunteers. This effect resulted from the zonula occludens toxin which leads to alterations in tight junction structure. With respect to viral causes of gastrointestinal infections, rotavirus has also been shown to exert an effect on epithelial barrier function which is thought to be mediated through different non-structural viral proteins.[31 32]

In our search for the structural basis of the barrier dysfunction in norovirus infection, an increased epithelial apoptosis and a reduction in tight junctional protein expression including occludin, claudin-4 and claudin-5 was observed. Both of the claudins are integral components of tight junction strands and have previously been shown to possess sealing properties. Overexpression of claudin-4 in Madin–Darby canine kidney (MDCK) cells in vitro decreased the paracellular conductance which was due to a selective decrease in sodium permeability.[33] From transfection experiments in MDCK cells, claudin-5 is also known to contribute to the barrier properties as a sealing tight junctional protein.[34 35] As mentioned, occludin was also found to be reduced in duodenal biopsies of norovirus infection. In a recent publication, in vitro infection with astrovirus, another diarrhoeagenic viral agent, led to barrier impairment along with a disruption in occludin staining of the tight junctional pattern.[36] Furthermore,

western blot analysis of rotavirus-infected intestinal cell lines demonstrated a reduction of the occludin signal.[37 38] However, the functional role of occludin regarding epithelial barrier function is still a matter of debate, since a direct investigation of functional barrier properties in occludin-deficient mice could not detect any change.[39] Despite barrier properties, a putative different role for occludin in viral infection comes from a recent publication of Coyne and co-workers which showed an occludin-dependent virus entry into enterocytes. Occludin is also internalised during viral entry and then subject to degradation. This would be a possible explanation for the disruption in occludin staining and the reduction in the total protein pool of occludin seen in different virus–enterocyte interactions including our own observation.[40]

Besides a reduction in sealing tight junction proteins, an increased epithelial apoptotic ratio was another prominent feature confirming the data from the gnotobiotic pig model.[14] While it was originally thought that epithelial apoptosis at the villus tip is a regular and highly sealed process without much barrier importance, our own group has presented direct experimental evidence that apoptoses represent leaks with a significant conductivity and therefore contribute to barrier dysfunction.[41] Apoptosis induction in norovirus infection could be mediated by different mechanisms. First, it might be caused by a direct virus-mediated effect, since in vitro data of an intestinal cell line showed apoptosis induction after transfection of the norovirus-encoded protein p20.[42] On the other hand, we found an elevated level of IELs, and most of them expressed perforin as a marker for activated cytotoxic T cells as discussed above. This indicates that a cell-mediated immune mechanism through cytotoxic T cells in response to the norovirus infection may play an additional role in enterocyte apoptosis induction. This mechanism has been proposed to be an important pathway in T cell-mediated gut injury.[43] In addition, there are data from experimental human norovirus infection and from the recently introduced gnotobiotic pig infection model showing high levels of interferon γ and tumour necrosis factor α, respectively, in this disease.[44] Therefore, release of cytokines could be another mechanism triggering the epithelial apoptosis.[41 45] Besides induction of apoptosis, there is compelling evidence for alterations in tight junction integrity mediated by proinflammatory cytokines through various mechanisms, including downregulation of sealing tight junction proteins.[46 47]

Regarding changes in epithelial net ion transport, the increased anion secretion was another novel finding in norovirus-infected small intestine. Besides the increased inhibitory current caused by NPPB and bumetanide, there was also an increased residual I_{sc} in the norovirus group. It is likely that this remaining current reflects further active anion transport that is not inhibited by NPPB and bumetanide. The reason for this incomplete inhibition in both groups is due to the fact that in bicarbonate-containing bathing solutions other anion transport mechanisms contribute to the overall rheogenic ion transport. Furthermore, in the human mucosa, inhibition of chloride secretion by bumetanide is not complete, strengthening the notion of increased chloride secretion as part of the remaining I_{sc}. In principle, anion secretion is an important pathomechanism in bacterial gastroenteritis leading to severe watery diarrhoea. The prototype is the cholera toxin-induced active electrogenic chloride secretion via an increase in intracellular cyclic AMP (cAMP).[48] Watery diarrhoea is also a prominent clinical feature in patients suffering from norovirus infection and besides the leak flux mechanism as a

result of the barrier dysfunction, anion secretion is the second important pathomechanism driving the diarrhoea. However, earlier studies of intestinal homogenates could not unequivocally show an increased level of cAMP.[49] Therefore, signalling pathways other than via increased cyclic monophosphates may be responsible for the changes observed in norovirus infection.

In conclusion, norovirus infection in humans leads to a combination of epithelial transport and barrier dysfunction. Duodenal specimens exhibited an increased anion secretion in combination with a severe epithelial barrier dysfunction due to downregulation of sealing tight junction proteins and increased epithelial apoptosis. Furthermore, we showed a reduction in villus surface area which was paralleled by the appearance of increased cytotoxic intraepithelial T cells.

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FOOTNOTES

Competing interests: None.

Ethics approval: The study was approved by the local ethics committee.

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